INTRODUCTION
Many tumor cells elicit procoagulant activity by interaction with the blood's thrombin-generating systems. It was generally resulted from Tissue Factor, which is expressed on the surface of the membrane. Thrombin, a serine protease, is a multifunctional protein to enhance tumor growth and pulmonary metastasis. Monocytes have a close relation to the bone metastasis as an osteoclast precursor. Activated monocytes express tissue factor and produce thrombin. In the present study, we confirm if monocytes express tissue factor by MDA-231 and report the process that argatroban inhibit bone metastasis of breast cancer cells (MDA-231) in vitro and in vivo.

METHODS
Monocyte isolation
Human peripheral monocytes were isolated by percoll gradient centrifugation using mono-poly resolving medium (Dainippon Seiyaku). The mononuclear cell layer was removed and mononuclear cell pellets were plated in 10-cm tissue culture in RPMI-1640 and 10% heat-inactivated fetal bovine serum (FBS). After 45 minutes at 37 degrees Celsius, adherent cells were collected by 0.5% EDTA for 30 min at 4 degrees Celsius as monocytes.

Analysis of Tissue Factor Expression
Cells were incubated with 1mg/ml anti-tissue factor antibody for 30 minutes. After washing, the cells were incubated with FITC-conjugated anti-rabbit IgG(1:100) for 30 minutes in the dark. After a final washing step, cells were analysed using a FACScan.

Osteoclast formation in coculture of mouse bone marrow cells and MDA-231
Bone marrow cells(4x10^5 cells/well) were isolated from 6-week old scid female mice and cocultured with the MDA-231 (2x10^5 cells/well) in 250 ml αMEM in 96-well plates. The cells were incubated with 0-10 μM of argatroban and 0.01μM of thrombin. After 7 days, the cells were stained for TRAP. The TRAP-positive multinucleated cells were counted as osteoclasts.

Effect of argatroban on bone resorption in vivo
MDA-231 cells (2x10^5) were injected into tibia of the mice. Argatroban was administered intraperitoneally at doses of 0 mg/kg/day for 28 days immediately after the inoculation of MDA-231 cells. Untreated mice received saline by intraperitoneal injections. After day 28, the bone resorption area of the tibia was calculated by degrees Celsius. The significance of the differences between argatroban-treated and untreated groups was estimated by student t-test using the STAT VIEW program.

Effect of argatroban on bone metastasis in vivo
MDA-231 cells (1x10^5) were injected into the left heart ventricle of the mice. Argatroban was administered intraperitoneally at doses of 9 mg/kg/day for 28 days immediately after the inoculation of MDA-231 cells. Untreated mice received saline by intraperitoneal injections. After day 28, incidence of bone metastasis was evaluated by radiography. The significance of the differences between argatroban-treated and untreated groups was estimated by student t-test using the STAT VIEW program.

RESULTS

**Tissue Factor expression of MDA-231**

**Tissue Factor expression of Monocyte**

**PREVENTION OF BONE METASTASIS BY THE THROMBIN INHIBITOR**

+*ASANUMA, K; *YOSHIKAWA, T; *WAKABAYASHI, H; **HAYASHI, T; *MATSUMINE, A; *KUSUZAKI, K; **SUZUKI, K; *UCHIDA, A
+Department of Orthopedic Surgery
Email: asanumaaa@aol.com
Department of Molecular Pathobiology
Mie University school of Medicine, Tsu City, Japan

**Fig 1. Tissue Factor expression**

MDA-231 was expressed Tissue Factor. FITC-labeled (green) and non-labeled (blue) cells were analysed (left). Before analysis of flow cytometry, monocytes were incubated with (green) or without MDA-231 culture fluid (blue) supernatant for 24 h including 10% PBS. Monocytes were stimulated Tissue Factor expression by MDA-231 culture supernatant (right).

**Fig 2. Osteoclast formation in vitro**

The co-culture between MDA-231(MDA), bone marrow(BM) and thrombin enhanced osteoclast formation(left). Proliferative stimulation of thrombin was inhibited by argatroban dose-dependently(right).

**Fig 3. In vivo study**

The limb bone resorption area in the argatroban group was decreased compared to the saline group (left). We showed the ratio of the metastasized bone number in each mouse (2 femur and 2 tibia). The metastasized limb bone number in the argatroban group was decreased compared to the saline group.

**DISCUSSION**

Many tumor cells express Tissue Factor including MDA-231 that we showed in this study. Tissue Factor activates factor X with factor VIIa, leading to the generation of thrombin. Activated monocytes are known to express Tissue Factor. We indicated that monocytes expressed Tissue Factor by MDA-231 culture supernatant, because it is believed to be caused by cytokines included in the MDA-231 supernatant. It is conceivable that the procoagulant activity of the MDA-231 metastasized area result from interaction with MDA-231 and monocytes. As thrombin is related with bone resorption to induce the differentiation from monocytes into mature osteoclasts in vitro, argatroban was effective on osteoclast formation in vitro and bone resorption in vivo. Furthermore, we showed anti-metastatic effect of argatroban. It was reported that the interaction between MDA-231 and monocytes enhances the malignancy. Monocytes not only have tissue factor and produce thrombin but have a close relation to osteoclastic genesis as a monocyte/macrophage precursor. Argatroban inhibited bone resorption and bone metastasis by reducing procoagulant activity and differentiation into osteoclasts.